REMARKS

The Office Action of September 10, 2002 presents the examination of claims 52-73 and 76-92. These claims are canceled herein, being replaced by new claims 83-120.

An interview with the Examiner was held on February 11, 2003. The cooperation of the Examiner in expediting prosecution of the present application is greatly appreciated.

Enablement

Claims 52-73 and 76-82 stand rejected under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement. This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

The Examiner takes a position that claims limited to the specific Ad-rsvLuc compositions presented as working examples in the specification are enabled, but that the broad scope of viral vector and any route of administration were not.

During the interview, Applicants explained that, as to lentivirus, adenovirus and similarly structured viruses, the viral elements important for the gene delivery function of the vector resided in the terminal repeat portions of the viral vector.

As discussed in the interview, the present claims recite that the vector is one derived from a lentivirus, adenovirus, adeno-associated virus or replication defective adenovirus. Such viral vectors have a general structure similar to that of the Ad-rsvLuc vector exemplified in the specification, at least as to the terminal repeat elements of the vector. Applicants submit that the present application fully enables claims of such scope.

The present method claims recite delivery by administration to the heart tissue or heart cavity, as expressly exemplified in the specification (see, Example 8). The claims also recite injection directly into the heart tissue. This is described incidentally by the procedure of Example 8. The Examiner will note the description at page 22, in which the injection needle is inserted into the heart tissue, then withdrawn until the location of the needle tip in the heart cavity is confirmed by the observation of blood in the syringe. The Examiner should further note expression of gene product (β-galactosidase) in cells along the needle track was observed (page 22, last paragraph).

The pending method of delivery claims further recite administration via injection into a blood vessel of the heart. Applicants submit that this route of administration is enabled in that the state of the art at the time the invention was made

recognized this as a way to deliver genes to heart tissue. The specification describes administration to the coronary artery, providing a reference for details, at the last paragraph of page 11. Also, during the interview, Applicants provided a copy of a paper (Tang et al. "Vigilant vectors: adeno-associated virus with a biosensor to seitch on amplified therapeutic genes in specific tissues in life-threatening diseases", *Methods* Vol. 28 (2002) pp. 259-266) that described administration of a recombinant vector via injection into either the jugular vein or the tongue vein with the result of cardiac-specific expression via the mlc-2 promoter. Applicants submit that, on the basis of the evidence of the specification and the Tang paper, enablement of delivery claims reciting administration via heart blood vessels is enabled.

The present claims also recite that the promoter is one that is the myosin light chain - 2 (mlc-2) promoter, or one comprising the elements of that promoter that confer cardiac-specific expression, such elements being operatively linked in the order in which they are found in a mammalian mlc-2 promoter.

The Examiner has also argued that, Applicants' evidence of unexpected results previously submitted to overcome rejections based on prior art illustrates the unpredictability in the art and, given such unpredictability, the claims must be limited in their scope. Applicants submit that the present claims recite

elements of vector structure that confer sufficient predictability to the use of the invention and that such elements furthermore are commensurate with the showing of unexpected results of record. Applicants submit that the presently pending claims are fully enabled by the disclosure of the specification, taken with what was known at the time the present invention was made. Accordingly, the instant rejection of claims 52-73 and 76-82 under 35 U.S.C. § 112, first paragraph, should be withdrawn.

Comments on the Interview Summary

For purposes of the interview of February 11, 2003, Applicants provided proposed claims to the Examiner. In the Interview Summary the Examiner comments that possible prior art issues are raised by the breadth of the term, "mlc promoter" in a "viral vector" in the proposed claims.

As to "mlc promoter", the present claims clarify that the promoter is an mlc-2 promoter, or the elements thereof that confer cardiac-specific expression. As to "viral vector, the Examiner will recall that Applicants' arguments of unexpected results were essentially that, at the time the invention was made, the skilled artisan had learned that some element present in the terminal repeats of adenoviruses abolished tissue specificity of expression from a promoter that was thought to be

tissue specific in its expression in some other context. The present invention overcomes that lack of tissue-specific expression that occurs when adenovirus or similar vectors are used for *in vivo* expression of a gene. Applicants submit that the currently presented claims recite structural elements of the vector that are commensurate with Applicants' explanation of the unexpected results achieved by the invention and the evidence of record supporting such explanation.

Applicants submit that the present application well describes and claims patentable subject matter. The favorable action of withdrawal of the standing rejections and allowance of the application is respectfully requested.

Pursuant to the provisions of 37 C.F.R. §§ 1.17 and 1.136(a), Applicants respectfully petitions for a four (4) month extension of time for filing a response from the due date of an Appeal Brief in connection with the present application. The required fee of $725.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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Attachment: Tang et al., Methods Vol. 28 (2002) pp. 259-266.

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METHODS

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Vigilant vectors: adeno-associated virus with a biosensor to switch on amplified therapeutic genes in specific tissues in life-threatening diseases

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Abstract

There are many life-threatening and chronic diseases in which physiological signals could be used to switch on therapeutic protective genes. We are developing a gene therapy approach in which a systemically injected "vigilant vector" waits for these signals and switches on genes to protect specific tissues with high amplification. The concept of a vigilant vector requires four components. The first component is a safe and stable vector that can be administered by systemic injection and express transgenes in a particular organ or tissue. The adeno-associated virus vector is safe and stable for this purpose. The second component is a reversible gene switch which is a biosensor that can detect certain physiological signals. We are developing a hypoxia switch, based on the oxygen-dependent degradation domain of hypoxia-inducible factor. The third component is a tissue-specific promoter, and we have used the myosin light-chain-2V promoter for specific expression in the heart. The fourth component is an amplification system. For this we have developed a double-plasmid/vector system based on the yeast GAL4 and human transcriptional activator p65 to produce a transactivating fusion protein that binds to a GAL4 activation sequence in an activating plasmid that then expresses high levels of cardioprotective genes.

Keywords: Vigilant vector; Adeno-associated virus; Cardioprotection; Hypoxia; Hypoxia-inducible factor; Myosin light-chain promoter; Double plasmid

1. Introduction

There are many life-threatening and chronic diseases in which physiological signals could be used to switch on therapeutic, protective genes. For example, repeated bouts of hypoxia in coronary artery disease lead to silent or overt myocardial tissue damage [1] and finally to heart failure. In diabetes the signal is high glucose; in cancer the signals are tumor markers. We are developing a gene therapy approach in which a systemically injected "vigilant vector" waits for these signals and switches on genes to protect specific tissues with high amplification [2]. The concept of a vigilant vector requires four components (Fig. 1). The first is a safe and stable vector that can be administered by systemic injection and express transgenes in a particular organ or tissue. The second is a reversible gene switch which is a biosensor that can detect a specific physiological signal. The third is a tissue-specific promoter that restricts transgene expression to the tissue or organ in danger. The fourth is an amplification system. We have found that the design is not obvious. A single vector containing the gene switch, promoter, and transgene does not, by itself, produce enough protective genes when the gene switch is on. This may be because of interference between the switch sequences and the promoter sequences. Therefore we have developed a double-plasmid (or vector) system to greatly amplify the transgene expression to provide the maximum protection.

To demonstrate how these components come together in an adeno-associated viral vector to make a vigilant vector, we have focused on designing the vector for use in protecting the heart during ischemia [2,3]. Therefore, the gene switch senses hypoxia and the promoter is specific in the heart.

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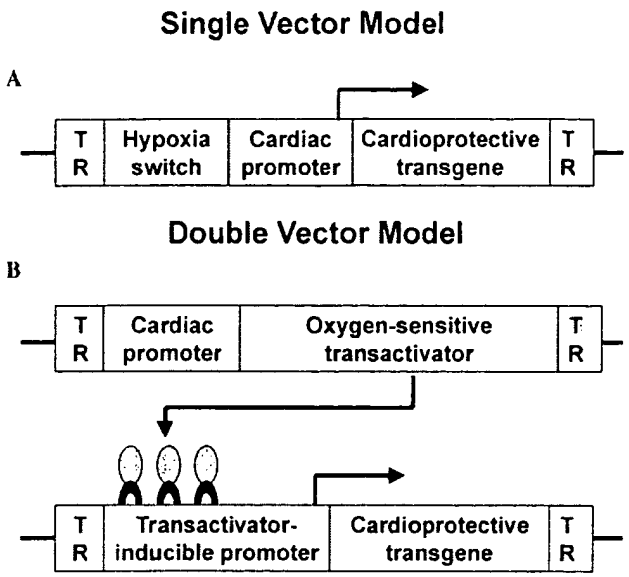

Fig. 1. Two models for the vigilant vector. (A) Single-vector model. Hypoxia switch, cardiac promoter, and cardioprotective transgene are adapted between the inverted terminal repeats (TR) of adeno-associated virus (AAV). (B) Double-vector model. In a single AAV, the cardiac promoter controls an oxygen-sensitive transactivator. The transactivator produces a fusion protein which binds to the inducible promoter and activates transcription of cardioprotective transgene in the second AAV.

1.1. Vector

For the vector, the recombinant adeno-associated virus (rAAV) is proving to be a stable, nonpathological vector [4,5]. Our data and data from other studies show that a single injection of rAAV (serotype 2) expresses transgenes in tissue for at least 6–18 months [5].

1.2. Gene switch/biosensor

The gene switch for the vector designed for protection against ischemia has to sense hypoxia. Hypoxia triggers a multifaceted adaptive response in mammalian cells, which is primarily mediated by a transcription factor termed hypoxia-inducible factor-1 (HIF-1) and its cognate DNA recognition site, known as hypoxia-response element (HRE) [6,7]. By utilizing HIF-1 or HRE, we have studied three models of hypoxic switch (Fig. 2).

1.2.1. HRE-containing promoter

To test the ability of HRE to induce transcription under hypoxia in cardiomyocytes, we tethered HRE to SV40 promoter or myosin light-chain-2 ventricular (MLC-2v) [8] promoter and put them upstream of a luciferase reporter gene (Fig. 2A).

1.2.2. Coexpression of HIF-1α with HRE/MLC-2v promoter

The protein level [9] and transactivity [10] of HIF-1α is inducible by hypoxia. Coexpressed HIF-1α may enforce the hypoxia-inducible ability of HRE.

1.2.3. Oxygen-sensitive chimeric transactivator

Under normal oxygen condition, the HIF-1α is undetectable [7] due to its rapid destruction by the ubiquitin–proteosome system [11–13]. But during hypoxia, HIF-1α is no longer degraded and accumulates exponentially as cellular O_2 decreases [9]. Within the HIF-1α, there is an oxygen-dependent degradation (ODD) domain (aa 401–603) [13–15]. It independently controls ubiquitin–proteosome mediated degradation of HIF-1α. The deletion of this entire region stabilized the HIF-1α. Conversely, the ODD domain alone confers oxygen-dependent instability when fused to a stable protein, Gal4.Thus, the vector contains an oxygen-sensitive chimeric transactivator (GAL4/ODD/p65) which was constructed by inserting ODD between the yeast GAL4 DNA binding domain and p65 activation domain.

1.3. Tissue specificity

Ubiquitous gene expression in all tissues may lead to undesired side effects in sites other than the target site of the gene therapy. To specifically express transgenes in the heart, we have studied the ventricular form of MLC-2v promoter. MLC-2v promoter is highly specific for the heart, during both embryonic and postnatal development [8] The MLC-2v promoter is 3.0 kb but the proximal 250-bp sequences, which contain the TATA box and several conserved *cis* regulatory sequences, HF-1 (HF-1a and HF-1b) [16–18], HF-2 [18,19], and HF-3, are sufficient to confer not only cardiac muscle specificity but also ventricular-restricted expression [19–23].

What is not obvious is that we could reduce the length of the MLC-2v by 3.0 kb, and still produce heart specificity. We tested the specificity of a 1700-kb MLC-2v promoter in rAAV in vivo and a 280-bp MLC-2v promoter in vitro.

1.4. Therapeutic genes

There are several genes that could be considered for protection of the heart during ischemia. In previous studies, we showed that antisenses to angiotensin II type-1 receptor [24,25], antisense to adrenergic β_1-receptor [26], or antisense to angiotensin-converting enzyme [27] protect rat hearts from ischemia–reperfusion injury. Others have shown that heme oxygenase-1 [28], which degrades the prooxidant heme and generates carbon monoxide and antioxidant bilirubin, confers myocardial protection from ischemia–reperfusion injury [8]. Superoxide dismutase protects the heart against superoxide radicals generated during ischemia–reperfusion [29,30]. Thus, these genes are good choices for cardioprotective transgenes in the vector.

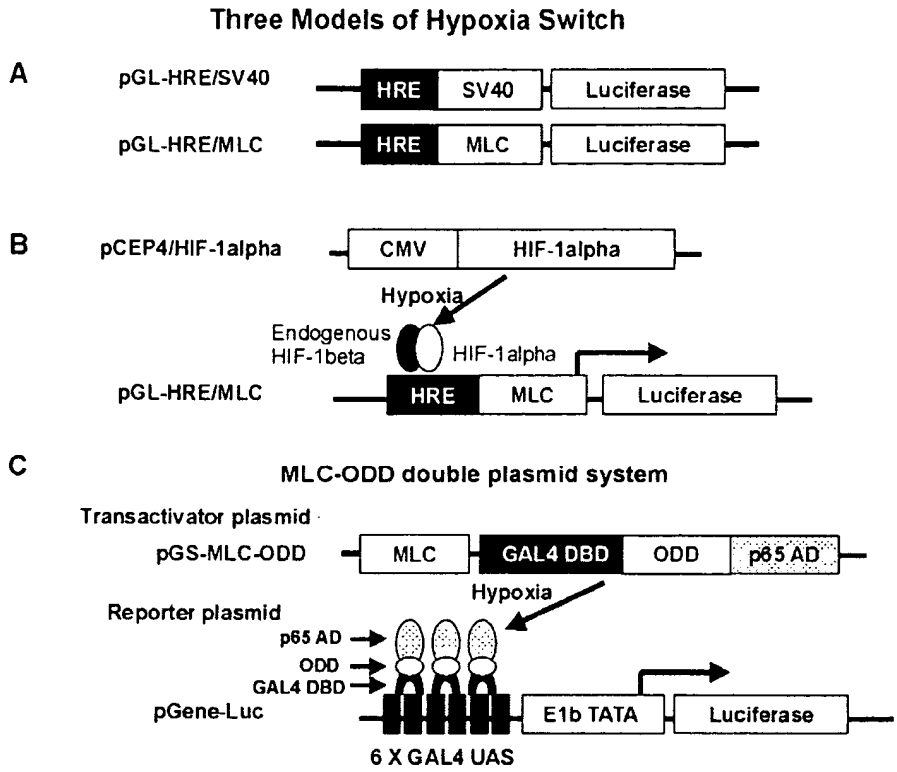

Fig. 2. Three models of a hypoxia switch. (A) Hypoxia-response element (HRE) has been inserted upstream of either the SV40 or the MLC-2v promoter to switch on luciferase expression in response to hypoxia. (B) Coexpression of HIF-1α-containing plasmid with the HRE/MLC-2v promoter. Under hypoxia the HIF- 1α subunit is stable and activated to dimerize the HIF-1β subunit. This binds to the HRE, stimulating transcription from the HRE/MLC-2v promoter. (C) Oxygen-sensitive chimeric transactivator (GAL4/ODD/p65) which is expressed by the transactivator plasmid under the control of the MLC-2v promoter. It can accumulate under hypoxia and activate the inducible promoter containing the GAL4 upstream activation sequence (UAS) in the reporter plasmid.

1.5. Transgene amplification

The working vigilant vector is not obvious. It may seem obvious to use a single plasmid or vector containing the required elements. However, when testing a single vector with the gene switch for hypoxia and an MLC-2v promoter, we found that there was not the expected increase in transgene expression at 1% O_2. Therefore, we developed an amplification system, using two plasmids (double-plasmid system) (Fig. 3), one for transactivation and another as the reporter. The transcriptional activator (GAL4/p65) consists of yeast GAL4 DNA binding domain and p65 activation domain [31]. When activated, the gene products form a fusion that binds to a GAL4 upstream activating sequence (UAS) inserted in the reporter plasmid and powerfully activates transcription. We tested the amplification effect of this system on MLC-2v, SV40, and cytomegalovirus (CMV) promoters. In addition to amplification, this double-plasmid system provides a convenient platform for the regulation of gene expression in response to hypoxia, for example, replacing the GAL4/p65 with a chimera containing the oxygen-sensitive gene switch, such as ODD (Fig. 2C) or using HRE/SV40 enhancer/promoter to control GAL4/p65.

2. Methods

2.1. Construction of plasmids and recombinant AAV

2.1.1. rAAV

Methods to package rAAV for systemic injection have been described previously [4,32].

2.1.2. PMLC-2v–green fluorescent protein (GFP)

We inserted a rat 1.7-kb MLC-2v promoter and the coding sequence of GFP between the inverted terminal repeats of AAV vector to construct pMLC-2v–GFP [2].

2.1.3. Gene switch plasmids

The plasmid, gene luciferase (pGL) with HRE and simian virus (SV) 40 promoter (pGL-HRE/SV40 [33]) was derived from pGL-SV40 (pGL2-Promoter, Promega, Madison, WI) by insertion of a 68-bp human enolase (ENO) 1 HRE sequence (−416 to −349, GenBank Accession No. X16287) into the 5′ flank of the SV40 promoter. The HRE sequence was also inserted into the 5′ flank of the MLC-2v promoter in the pGL-MLC to generate pGL-HRE/MLC [2].

pCEP4/HIF-1α [34], which contains human HIF-1α cDNA sequence downstream of a cytomegalovirus

promoter, was a gift from Dr. Gregg L. Semenza (Johns Hopkins University, Baltimore, MD).

We have developed the use of the ODD domain as a gene switch for hypoxia. ODD (amino acids 394–603) [13] was amplified by PCR from pCEP4/HIF-1α [33] and inserted in-frame between the coding sequence of GAL4 DNA binding domain and p65 activation domain (GAL4/ODD/p65) in pGS-MLC to generate pGS-MLC-ODD. The key to the invention, which is not obvious, is the insertion of ODD between GAL4 and p65 which confers a powerful O_2 switch action when linked to a promoter.

2.1.4. Transgene amplification plasmids

Single plasmid system. The single plasmid system is shown in Fig. 3A. Three promoters have been inserted in front of the transgene which in this case is a luciferase reporter gene. pGL-SV40 (Promega, CA) contains SV40 promoter. pMLC-Luc harbors a 281-bp (−264 to 17, GenBank Accession No. U26708) fragment of MLC-2v-promoter. pCMV-Luc has CMV enhancer and promoter.

Double-plasmid system. To activate a highly amplified transgene expression, the double-plasmid system (Fig. 3B) is used. For the transactivator plasmid, pGS-CMV, a generous gift from Dr. Sean M Sullivan (University of Florida, Gainesville, FL), expresses a chimeric transcription factor consisting of the yeast GAL4 DNA binding domain (amino acids 1–93) [35] and the human p65 activation domain (amino acids 283–551) [36] from NF-κB under the control of a CMV enhancer/promoter. The CMV promoter has been replaced with an SV40 promoter, HRE/SV40 enhancer/promoter, or 281-bp MLC-2v promoter to generate pGS-SV40, pGS- HRE/SV40, and pGS-MLC, respectively. For the reporter plasmid, pGene-Luc encodes luciferase driven by six copies of a 17-bp GAL4 UAS [37] and an adenovirus-derived E1b TATA box [38]. It was derived from pGene/V5-His/*lacZ* (Invitrogen, CA) by replacing the *lacZ* coding sequence with a luciferase cDNA. The identity of clones was confirmed by nucleotide sequence analysis.

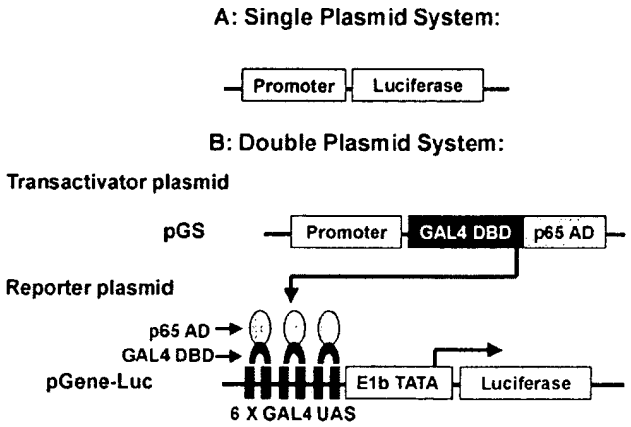

Fig. 3. The amplification system. (A) A single-plasmid system is the traditional way to express transgene, in which a promoter drives transgene (luciferase) expression directly in one plasmid. However, this does not produce enough transgene. (B) A double-plasmid system can amplify the power of promoter based on the strong transcription activity of GAL4/p65 fusion protein. The promoters in the transactivator plasmid (pGS) tested included SV40, HRE/SV40, CMV, and MLC-2v. The reporter plasmid contains GAL4 upstream activation sequence (UAS) in front of an adenovirus E1b TATA box and the firefly luciferase reporter gene. The fusion protein binds to the GAL4 UAS and promotes an amplified transgene expression.

2.2. Testing the plasmids in vitro

2.2.1. Cell culture and hypoxic treatment

A rat embryonic cardiac myoblast cell line, H9c2 (CRL1446; ATCC, Monassus, VA), was maintained in Dulbecco's modified Eagle's medium supplemented with sodium pyruvate and 10% fetal bovine serum. Cells were cultured under normoxic conditions (5% CO_2, 20% O_2, 75% N_2, v/v) in a humidified incubator at 37 °C. For hypoxic treatment, cells were put into hypoxia chambers (Oxygen Sensors). The chambers were connected to a tank with premixed gas (1% O_2, 5% CO_2, and 94% N_2) at one end and to a vacuum at the other end. Hypoxia condition was achieved by evacuating and gassing the chambers for six times. Then the tightly sealed chambers were incubated at 37 °C. The oxygen level in the chamber and medium is around 7.6 mm Hg, which was monitored by an OxyLite probe (Oxford Optronix, UK).

2.2.2. Transient transfection and reporter gene assays

Cells were transfected at a confluence of 50–60%. Transfection was performed with LipofectAMINE (Invitrogen) according to manufacturer's protocol. pRL-CMV (Promega) or pRL-TK (Promega) coding *Renilla* luciferase was used to control transfection efficiency. Luciferase assays were performed with dual luciferase assay system (Promega). Results were quantified with a Monolight 3010 luminometer (Pharmingen) and expressed as a ratio of firefly luciferase activity over *Renilla* luciferase activity.

2.3. Routes of injection for system delivery of AAV-based vectors

There are three routes of injection that we have used for injecting the vigilant vectors and recombinant AAV in vivo. The methods apply to young rats or mice.

2.3.1. Jugular vein

With the mouse in supine position, the jugular vein can be detected just under the neck skin by pulsation accompanying the respiratory rhythm. Using a 30-gauge needle, the vector is injected slowly in a volume of 50 μl. The advantage of the jugular route is that it has large capacity for relatively large volumes and can done without surgery.

2.3.2. Tongue vein injection

Under light anesthesia, the tongue is pulled forward, revealing the tongue vein. Again, using a 30-gauge needle, the vector is injected slowly in a volume of no more than 50 μl. The advantage of this method is that the vein is accessible without opening skin, and the injectate can be seen entering the circulation.

2.3.3. Intracardiac injection

For intracardiac injection the animal is lightly anesthetized with an inhalant such as metofan or isoflurene and placed on its back. The needle of the syringe is injected under the xiaphoid process angle slightly left to enter the left ventricular chamber. The syringe is drawn back until blood is seen and then the injectate is injected directly into blood. The advantage of this method is that it provides direct injections that are rapidly distributed through the body. In our experience with this method of injection [39], we have not seen any toxic response or infection.

3. Results

3.1. The cardiac specificity of MLC-2v promoter in vitro and in vivo

The pGL-MLC was specifically expressed in cardiomyocytes. After transient transfection, the luciferase expression in cardiomyocytes (H9c2) is 29.38 ± 13.11-fold higher than that in rat glioma cells (C6) [2] and 20.07 ± 1.71-fold higher than that in pulmonary vein endothelial cells. The transfection efficiency in three cell lines was normalized by the expression of an internal control plasmid containing ubiquitous viral promoter (CMV or thymidine kinase promoter).

3.2. The cardiac specificity of MLC-2v promoter in vivo

Four weeks after a systemic injection into adult mouse or 5-day-old rats, the transduction of rAAV-MLC-2v-GFP was shown by PCR of DNA in many tissues, such as spleen, liver, lung, kidney, and heart. The tissue-specific expression of GFP mRNA under MLC-2v promoter was examined by RT-PCR and was detected only in heart [2]. The presence of GFP protein was further examined by immunofluorescence staining, which was apparent in the heart of the treated animal and absent in the control animal (injected with saline). GFP was undetectable in the kidney and liver of the same treated animals and controls [2].

3.3. The hypoxia-inducible ability of three designs of gene switches

H9c2 cells were transfected with pGL-HRE/SV40 alone, pGL-HRE/MLC alone, or both HIF-1α and pGL-HRE/MLC and then subjected to 1% O_2 for 24 h (Fig. 4). The luciferase expression of HRE/SV40 (Fig. 4A) construct increased by 7.12 ± 1.52-fold in cells incubated under hypoxia relative to cells incubated under 20% O_2. However, pGL-HRE/MLC did not show significant hypoxic induction. In the presence of overexpressed HIF-1α, HRE/MLC showed 3–4-fold increases under hypoxia (Fig. 4B).

The MLC-ODD double-plasmid system has also been tested under 1% O_2 for 24 h (Fig. 5). In H9c2 cells this system showed 5.75 ± 1.38-fold increase of expression. This system worked even better in the primary

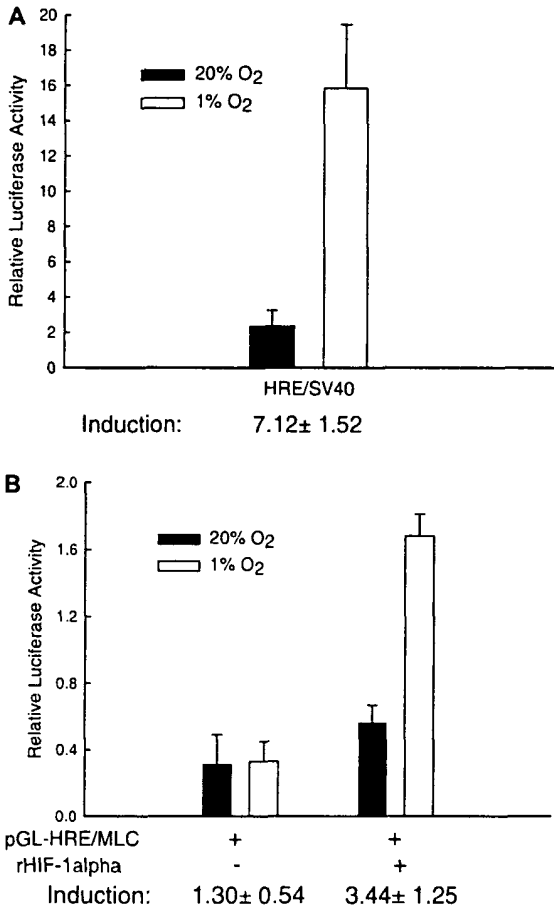

Fig. 4. The hypoxia-inducible ability of HRE-containing gene switch. (A) HRE can switch on SV40 promoter in response to 1% O_2; 2 μg/well pGL-HRE/SV40 was transfected into H9c2 cells along with 100 ng/well pRL-TK control plasmid in 60-mm dishes. Duplicate plates were incubated in either 20 or 1% O_2 for 24 h before preparation of cell lysates. Results are expressed as a ratio of firefly luciferase activity over *Renilla* luciferase (relative luciferase activity). The ratio of relative luciferase activity in cells at 1% O_2 compared to 20% O_2 was also calculated to determine induction by hypoxia (mean ± SD; $n = 3$ independent experiments). (B) HRE/MLC-2v enhancer/promoter alone could not augment luciferase expression under hypoxia. However, in the presence of rHIF-1α, HRE/MLC-2v could increase luciferase expression by 3–4-fold in response to hypoxia. H9c2 cells were cotransfected with 2 μg/well pGL-HRE/MLC and 100 ng/well pRL-TK, in the absence or the presence of rHIF-1α. The cells were exposed to 1 or 20% O_2 for 24 h (mean ± SD; $n = 4$ independent samples).

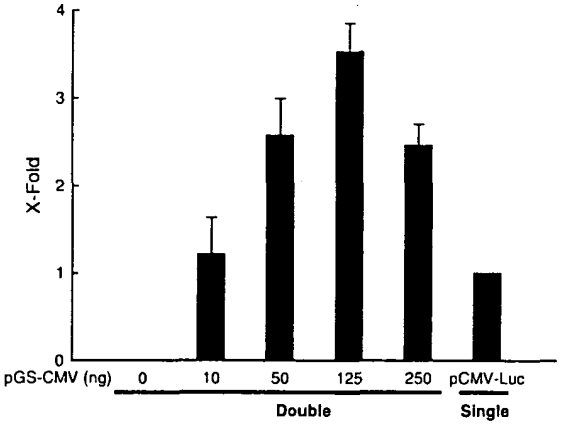

Fig. 5. The double-plasmid system amplified the power of the CMV promoter. For double-plasmid system transfection, H9c2 cells seeded in six-well plates received 125 ng/well control pRL-TK, 250 ng/well reporter plasmid (pGene-Luc), 0–250 ng/well transactivator plasmid (pGS-CMV), and various amounts of empty vector such that all cells received a total of 625 ng plasmid per well. The transfection of 250 ng/well pCMV-Luc served as the single plasmid system. The relative luciferase activity from double-plasmid transfection was normalized to that obtained from the single-plasmid system (X-Fold) (mean ± SD; $n = 3$ independent samples).

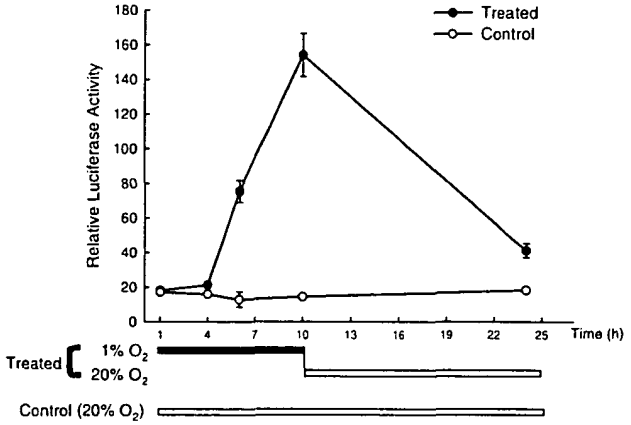

Fig. 6. The switch on-and-off function of the HRE/SV40 double-plasmid system. H9c2 cells were transfected with 10 ng/well pGS-HRE/SV40 and 2 μg/well pGene-Luc. The cells in the treated group were first exposed at 1% O_2 for 10 h and then returned to 20% O_2 for another 14 h. Thus, the luciferase expression was first increased during the hypoxic period, then dropped down after reoxygenation. The cells in the control group were continuously incubated at 20% O_2 (mean ± SD; $n = 3$–6 independent samples).

culture of adult rat cardiomyocyte, with the hypoxia induction ratio at 17.89 ± 4.85. However, without ODD, GAL4/p65 fusion protein did not significantly increase reporter expression.

3.4. The amplification effect of double-plasmid system on MLC-2v, HRE/SV40, SV40, and CMV promoters

For a strong promoter, such as CMV, the double-plasmid system could increase the power of CMV promoter up to 3.52 ± 0.33-fold over that of the single plasmid (Fig. 5). For a relatively weak promoter, such as SV40, there was a 410.56 ± 84.42-fold increase [3]. For a tissue-specific promoter, such as MLC-2v, the increase was 346.08 ± 22.50-fold and without compromising cardiac specificity expression. Although by adding a higher dose of transactivator plasmid we could further increase the expression level of the MLC double-plasmid system, the tissue specificity was decreased. The double-plasmid system also effectively increased the level of HRE/SV40 promoter up to 412.79 ± 185.27-fold at 1% O_2 and 205.35 ± 65.44-fold at 20% O_2 and still maintained its hypoxic induction ability [3]. The HRE/SV40 double-plasmid system started increasing expression at 4 h, this increase is reversible (Fig. 6).

4. Time of onset

While we do not know the time of onset of gene activity in vivo, we have tested time of onset in vitro. Careful measurements of O_2 levels in the cultures have revealed that it takes 2.6–3.8 h after O_2 has been reduced from 20 to 1% in a closed chamber to reach 1% in the culture medium. The reporter gene in vector in this cell culture system was significantly higher at 4–6 h. Therefore, subtracting the time to reach 1% in the media, the activation of the system is about 0.2–1.4 h. The time for therapeutic genes to act is within this time frame except in absolute anoxia.

5. Discussion

In conventional gene therapy strategies, the expression of transgenes is constitutive. This produces an unphysiological expression pattern of protein production which could lead to several deleterious consequences. These include inappropriate downregulation of effector systems, cellular toxicity, and pathophysiology [40,41]. Therefore, being able to switch on or off a vector is a highly desirable aspect for gene therapy. Several inducible gene expression systems have been proposed. These systems are usually composed of two or three coexpressed vectors. One vector (in some cases there are two) expresses a chimeric transcription factor, which functions as a gene switch. This switch is regulated by the administration of a small molecule. The other vector contains reporter gene downstream of the switch-controllable promoter. These systems include the following: (1) The tet on–off system, a tetracycline (tet)-regulated system, is either a tet-repressible or a tet-activating system. In the presence of antibiotic, tetracycline, a tet-repressor containing transactivator (tTA) does not bind to a tTA-dependent promoter and prevents transcription. By withdrawing tet, the system is turned on [42]. In

the tet-activating system, the transactivator contains a mutant tet repressor that requires doxycycline, a tet derivative, for specific DNA binding. Thus, the system is turned on in the presence of doxycycline and turned off without doxycycline [43]. (2) The ecdysone-inducible system [44] uses three plasmids. Two plasmids express retinoid X receptor and ecdysone receptor/VP16 fusion protein. Ecdysone binds to and activates the ecdysone receptor/VP16. The activated fusion protein and retinoid X receptor will heterodimerize and bind to ecdysone response element in the presence of muristerone A, which is a synthetic analog of ecdysone, to transactivate transcription. (3) The antiprogestin-regulated gene switch is a transactivator consisting of the truncated human progesterone receptor ligand- binding domain in the middle of the VP16 transactivation domain and the yeast GAL4 DNA- binding domain. This fusion protein activates GAL4 binding site contained promoter in response to progesterone antagonists [45], such as RU486/mifeprostone [46]. (4) The dimerization-based gene switch system coexpresses two fusion proteins, one consisting of FKBP and DNA-binding domain and the other containing FRAP and the transactivation domains [47]. These two fusion proteins are associated by rapamycin through FKBP and FRAP. The heterodimeric protein complex acts as a transcriptional activator to activate transcription from an inducible promoter.

The weakness of all of these regulable systems is that they need exogenous drugs as inducers. Not only does this produce unwanted side effects, but it also defeats the purpose of gene therapy which has to be highly specific and nontoxic. For example, tetracycline deposits in bone and stains teeth [48]. Long-term administration may lead to bacteria resistance. RU486/mifeprostone is a progesterone antagonist [45]. Rapamycin has growth-inhibitory and -immunosuppressive effects.

Therefore, the vigilant vector is designed to respond directly to endogenous pathophysiological signals, such as hypoxia and to switch off when normotoxia returns without any drug being given. HRE alone as a hypoxic gene switch, fused to either a similar virus (SV40) promoter [49,33] or a CMV promoter [50], has been reported to successfully increase the gene expression in response to hypoxia. We have obtained similar positive results when we tested HRE/SV40 promoter in cardiomyocytes [3]. However, under hypoxia, HRE alone was not able to upregulate transcription from a tissue-specific promoter, MLC-2v promoter [2], in cardiomyocyte cultures. Although overexpressed HIF-1α could help HRE to regulate the MLC-2v promoter, the expression level was quite low. We designed the oxygen-sensitive transactivator and put it under the control of the MLC-2v promoter. This has proven to be a better way to construct a tissue- specific and hypoxic-inducible vector.

According to the results of other groups [51] and ours, tissue-specific promoters are relatively weak compared to nonspecific promoters, such as CMV. The challenge is to increase the promoter strength without losing specificity. Several methods have been tried to circumvent the power limitation of the MLC-2v promoter. An SV40 enhancer can increase the activity of MLC-2v promoter by at least 10-fold, but it decreases its cardiac specificity [52]. Although three copies of the HF-1, HF-2, and HF-3 elements did not compromise the specificity of the MLC-2v promoter [2], there was only a 3-fold increase in activity. Overexpression of heart factor-1b leads to just a 2–3-fold increase in the activity of the MLC-2v promoter [2]. The double-plasmid method dramatically increased the power of the MLC-2v promoter by more than 300-fold and still maintained its specificity. This method could be applied to other tissue-specific promoters

Our strategy needs two plasmids to cooperate, and in the context of rAAV, it will be a double-virus system. This raises the question of whether two viruses can enter the same cell. Several methods using two rAAVs to overcome its size limitation provide supporting evidence for our strategy. Relying on the intrinsic property of rAAV to undergo intermolecular concatamerization [53,54], one approach [55] has used a vector encoding multiple enhancer sequences to increase the transgene expression in a second, coinfected transgene-containing vector. The other [56] is to split a large gene into two parts and separately package them into two individual rAAV vectors. After being coinfected into target cells, these two rAAV vectors form head-to-tail heterodimers and restore the coding sequence of the transgene after RNA splicing. Therefore, there are solutions for two rAAVs to enter the same cell and work together should the simple empirical test of injecting two vectors at the same time prove inadequate.

The cardioprotective genes listed in the introduction protect ischemic heart through different mechanisms. We have used heme oxygenase-1 for ischemia and superoxide dismutase for reperfusion protection. Thus, to provide full protection, cotransfer of multiple protective genes may be necessary. For example, a rat model of Parkinson's disease coexpression of the L-dopa synthesizing enzyme, tyrosine hydroxylase, and its cofactor synthetic enzyme, GTP-cyclohydrolase-1, by rAAV provided substantial functional improvement [57]. However, the effects after delivery of the tyrosine hydroxylase gene alone have been disappointing. Thus, the double-virus system could be expanded to a triple- or more-virus system, with one transactivator virus expressing an oxygen-sensitive and strong regulator and several other viruses carrying different protective genes under the inducible promoter.

The concept of a vigilant vector has broad applicability. By switching the tissue-specific promoters, gene switches, and protective genes, this concept can be developed and applied generally to a number of other

disease states. Among these are diabetes type 1 where glucose would switch on preproinsulin genes, cancer where tumor markers could switch on antigrowth and antiangiogenic genes, and stroke where hypoxia could switch on tPA. One could further imagine that a vigilant vector could lie in wait to protect against the cellular effects of biological warfare attacks, inhibiting for example, the uptake of anthrax toxin.

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